



DOCKET NO. 3802-126-27 CONT

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

IN RE APPLICATION OF: De-Chao YU, et al.

Art Unit: 1633

SERIAL NO.: 10/691,045

Examiner: Maria Marvich

FILING DATE: October 21, 2003

FOR: CELL-SPECIFIC ADENOVIRUS VECTORS COMPRISING AN  
INTERNAL RIBOSOME ENTRY SITE

**AFFIDAVIT OF PRIOR INVENTION UNDER 37 CFR 1.131**

Commissioner for Patents  
P.O. Box 1450  
Alexandria, VA 22313-1450

Dear Sir:

I, DE-CHAO YU, being duly sworn, depose and state:

1. This Declaration is submitted as evidence that the subject matter claimed in the above-identified application was reduced to practice by the present inventors prior to the publication of Chang et al (WO 99/25860).
2. I understand that the Patent Application was filed on October 21, 2003 with the United States Patent & Trademark Office by Piper Rudnick, and was accorded Serial No. 10/691,045.
3. Andrew Little and I are co-applicants of the above-identified patent application, and the inventors of the subject matter described and claimed therein.
4. Throughout the time period from 1999 to 2001, I was Vice President of Research at Calydon Corporation and was involved in and supervised all projects directed to development of adenovirus vectors, more specifically, Attenuated Replication-Competent Adenovirus ("ARCA") vectors for local and systemic treatment of cancer. One project

of cancer. One project that I supervised was specifically directed to development of ARCA vectors comprising an internal ribosome entry site or "IRES".

5. In my role as Vice President of Research at Calydon Corporation, I supervised the work of Andrew Little who supervised the technical activities performed by Melissa Scott on the project directed to development of ARCA vectors comprising an internal ribosome entry site or "IRES".
6. This Declaration is submitted as evidence that the subject matter claimed in the above-identified application was reduced to practice by the inventors of the instant application prior to the publication of Chang et al. as explained below.
7. I have reviewed the presently pending claims numbered 59-88, as set forth in the amendments dated June 15, 2005 and October 12, 2005.
8. Prior to the publication of Chang et al (WO 99/25860; published May 27, 1999), the inventors of the above-identified application conceived the invention as claimed in at least Claims 59-88 of U.S. Application Serial No. 10/691,045, as noted herein with respect to Exhibits 1-8, which are all dated prior to May 27, 1999. Note that the dates have been redacted from Exhibits 1-8.
9. Page 70-71 of Cell Genesys Notebook # 96 issued to Andrew Little, provides a copy of a memo to me, "DC" from Andrew Little "Andy" (Exhibit 1). The subject matter of the memo is "IRES Strategy" and it is dated December 31, 1998. The memo describes the IRES strategy that was implemented in the experiments summarized in the subsequent pages of notebooks issued to Andrew Little and Melissa Scott. The memo states that the purpose of the project is to regulate expression of both the E1a and E1b genes using a single tissue specific promoter. Both the ECMV and VEGF IRES are referred to in the memo.
10. The memo makes reference to a strategy for cloning a platform plasmid for constructing Ad mutants that express IRESs. The cloning strategy is summarized in a hand written diagram found on page 72 of Genesys Notebook # 96 issued to Andrew Little (Exhibit 2).

11. The experimental details for cloning the platform plasmid and constructing Ad mutants that express IRESs are found on pages 73-117 of Cell Genesys Notebook # 96 issued to Andrew Little (Exhibit 3). The notebook pages describe the construction of two vectors in which the expression of the adenoviral E1a gene is under operative control of either a probasin or CMV promoter and expression of the adenoviral E1b gene is under operative control of an ECMV IRES.
12. Page 117 of Cell Genesys Notebook # 96 states "cloning turned over to Melissa Scott". (Exhibit 3).
13. Page 114 of Cell Genesys Notebook # 99, issued to Melissa Scott states that the goal of the experiment is to regulate expression of both the E1A and the E1B gene using a single tissue specific promoter and that to do so an internal ribosome entry site or (IRES, an internal entry point for initiation of translation by eukaryotic ribosomes) must be cloned between the 2 genes. Both the EMCV and VEG-F IRESs are described. Page 114 further states "Picking up project in the beginning.....where Andy left off" (Exhibit 4).
14. Pages 115-119 of Cell Genesys Notebook # 99, summarize the construction of an adenoviral platform plasmid which has the E1A and E1B genes separated by an EMCV IRES and use of the platform plasmid to make adenoviral vectors which include two different promoters, the probasin ("PB") or cytomegalovirus ("CMV") promoter (Exhibit 5). The experiments detailed by Melissa on pages 116-119 confirm the presence of the IRES in two clones prepared by Andrew Little.
15. The experiments detailed by Melissa Scott on pages 119 and 121 of Cell Genesys Notebook # 99 (Exhibit 6), describe addition of a CMV promoter to the adenoviral platform plasmid. The experiments detailed by Melissa on page 120 and describe addition of a probasin promoter to the adenoviral platform plasmid. (Exhibit 6).
16. Pages 133-134 of Cell Genesys Notebook # 99, issued to Melissa Scott summarize the analysis of constructs and confirmation of the insertion of the CMV and probasin

promoters (Exhibit 7). Five out of eight constructs were demonstrated to have the promoter in the correct orientation (page \*\*\*; Exhibit 8).

17. It should be noted that any date blocked out in Exhibits 1-8 were prior to the publication date of the Chang et al. (WO 99/25860), published May 27, 1999.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Signed at San Francisco, California, this 2<sup>nd</sup> day of May, 2005<sup>6</sup>

DE-CHAO YU

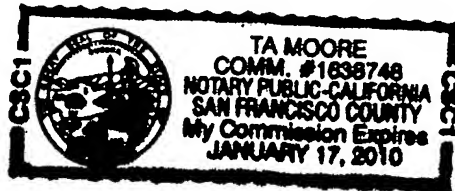


STATE OF California :

COUNTY OF San Francisco:

Before me, a Notary Public for said County, personally appeared DE-CHAO YU, known to me to be the person who executed the foregoing affidavit and acknowledged it to be his act and deed.

Witness my hand and seal this 2<sup>nd</sup> day of May, 2005<sup>6</sup> J. Moore



From Page No. 1



TO: DC  
FROM: ANDY  
SUBJECT: IRES STRATEGY  
DATE:  
CC:

## INTRODUCTION

The purpose of the project is to regulate the expression of both the E1a gene and the E1b gene using a single tissue specific promoter. One approach is to engineer both genes to be expressed in one mRNA. To insure efficient translation of the second cistron, an internal ribosome entry site will be cloned between the two genes. After reviewing relevant literature, I determined that two IRESes may be suitable. The first IRES is from encephelomyocarditis virus (EMCV). The EMCV IRES has been well characterized. Researches have delineated the essential bases, and it is commercially available from Novagen (Madison, WI). Others have used the EMCV IRES in gene therapy studies using replication defective retroviruses and adenoviruses. The second IRES selected is from the mouse VEG-F gene. Several genes that regulate cell proliferation and promote cell transformation, like fibroblast growth factor and insulin growth factor, contain IRESes. One hypothesis for why cellular proliferation genes contain IRESes is that cap-dependent translation is not efficient under hypoxic conditions. Huez et al. (1998) recently described a truncated 163 base IRES from a differentially spliced VEG-F mRNA. Their work showed that this IRES was more active than the full length VEG-F IRES and had activity in human cells. A human VEG-F IRES was also recently described. However, it has two independent elements that total 700 bases.

## PLATFORM PLASMID CONSTRUCTION

I have attached a strategy for cloning a platform plasmid for constructing Ad mutants that contain IRESes. The first step is to create a deletion in CN306, a plasmid derived from pXC.1 lacking the E1a promoter, that removes the E1B promoter and E1a poly A signal, but leaves the E1a and E1b coding regions intact. This can be accomplished by overlap PCR (see attached). Primers A and C amplify a 288 bp fragment, and primers B and D amplify a 396 bp fragment. Primers C and D were designed to contain 15 b of homology to each other so that the two PCR products could be annealed. This 15 bp region of homology also contains a unique Sal I site that will be

To Page No. 7

Witnessed &amp; Understood by me,

  
Christine M. M.

Invented by

Recorded by

used to clone in the IRESes. The Sal I site has been engineered so that IRES inserts will be placed 6 bases (or 2 amino acids) upstream of the E1b translation initiation codon. The overlap product (669 bp) will then be digested with Xba I and Kpn I and cloned into similarly cut CN306. All together, these manipulations delete about 118 bp of endogenous Ad sequence. Once the platform plasmid has been engineered, viruses can be generated by homologous recombination with Ad right hand end plasmids in 293 cells.

#### EMCV IRES

The EMCV IRES can be cloned from a commercially available plasmid (pCITE-3) using PCR. I suggest using pCITE-3 as a template because the IRES has been modified for optimal performance. Primers E and F amplify a 519 bp IRES fragment with Sal I ends that can be ligated into the platform plasmid described above. The EMCV IRES contains a pyrimidine rich tract of bases near its 3' end that are important for its activity. This pyrimidine tract will be 27 bases (9 amino acids) from the E1b initiation codon.

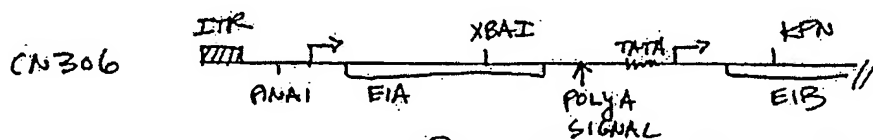
#### VEG-F IRES

Since the VEG-F IRES is only 163 bases, it may be possible to synthesize it with Sal I linkers. Because this IRES is from an alternatively spliced mRNA and cloning it would require several steps, synthesizing it may save us valuable time. The proposed sequence is attached. Once synthesized, the IRES could be easily cloned into the platform described above.

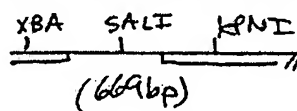
#### INTELLECTUAL PROPERTY ISSUES

One issue that needs to be considered before beginning the project is if patents applications have been filed on any of these IRESes. If so, do we need to license the IRESes? Or, do we proceed anyway to assess project feasibility before negotiating licensing terms? I suggest that we do a comprehensive intellectual property search.

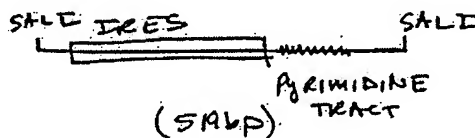


PLATFORM PLASMID CONSTRUCTION

GENERATE OVERLAP PRODUCT TO CREATE  
DELETION OF EIB PROMOTER ETC.



OVERLAP PRODUCT CAN BE CLONED  
DIRECTLY BACK INTO CN306 TO CREATE  
DESIRED PLATFORM PLASMID WITH  
UNIQUE SAL I SITE.

EMCV IRES

BEST AVAILABLE COPY

Reviewed & Understood by me,

*[Signature]*

Invented by

Recorded by

*[Signature]*

## BEST AVAILABLE COPY

VEGF IRES

## SENSE PRIMER

5' <sup>SAL I</sup> ACGTAGTCGACAGCGCAGAGGCTTGGGGCAGCCGAGCGGCAGCCA  
GGCCCCGGCCCCGGGCTCGGTTCAGAAAGGGAGAGGAGCCCGCCAAGGCG  
CGCAAGAGAGCGGGCTGCCTCGCAGTCCGAGCCGGAGAGGGAGCGCGAGC 1856  
CGCGCCGGCCCCGGACGGCCTCCGAAACCA<sup>SAL I</sup>TGTCGAC ACGTA 3'

## ANTISENSE PRIMER

5' <sup>SAL I</sup> TACGTGTCGACCATGGTTTCGGAGGCCGTCCGGGGCCGGCGGGCTCGCG  
CTCCCTCTCCGGCTCGGACTGCGAGGCAGCCCGCTCTCTTGC CGGCCTTGG 1856  
CGGGCTCCTCTCCCTTCTGGAACCGAGGCCCGGGCCGGGGCTGGCTGCCG  
CTCGGCTGCCCAAGCCTCTGCGCTGTGCACTACGT 3' <sup>SAL I</sup>

THESE TWO OLIGOS WILL BE ANNEALED  
AND CLONED INTO UNIQUE SAL I SITE OF  
PLATFORM PLASMID.

NOTE - AFTER DISCUSSION WITH  
KEYSTONE, I WILL DIVIDE EACH ABOVE  
OLIGO IN TWO AND SYNTHESIZE BY ~906  
OLIGOS INSTEAD OF 2 ~1806 OLIGOS.  
APPARENTLY, LONGER OLIGOS CAN CONTAIN  
DELETIONS/INSERTIONS.

*[Signature]*

*[Signature]*



Page No. 73

IRES CLONING OLIGOS

Amplification of EMCV IRES from Novagen pCITE-3a(+) vector

Sense

(96.74.1) GAC GTC GAC ATC GTG TTT TTC AAA GGA A (pCITE sequence 9 to 27) 28

Antisense

(2) GAC GTC GAC TAA TTC CGG TTA TTT TCC A (pCITE sequence 491 to 509) 28

Amplification of E1a/E1b intergenic region

Sense

Primer A

(3) CCT GAG ACG CCC GAC ATC ACC TGT G (Ad sequence 1314 to 1338) 25

Primer D

(4) TGC TGA ATG GTC GAC ATG GAG GCT TGG GAG (Ad sequence 1714 to 1728) 30

Antisense

Primer B

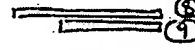
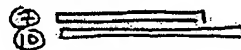
(5) CAC AAC CGC TCT CCA CAG ATG CAT G (Ad sequence 2070 to 2094) 25

Primer C

(6) GTC GAC CAT TCA GCA AAC AAA GGC GTT AAC (Ad sequence 1572 to 1586) 30

Mouse VEG-F oligos to reconstruct SP163

Sense: primers



PRIMER  
ATTACHING

(7) ACG TAG TCG ACA GCG CAG AGG CTT GGG GCA GCC GAG CGG CAG CCA  
GGC CCC GGC CCG GGC CTC GGT TCC AGA AGG GAG AGG AGC CCG CCA

90mer

(8) AGG CGC GCA AGA GAG CGG GCT GCC TCG CAG TCC GAG CCG GAG AGG GAG CGC GAG  
CCG CGC CGG CCC CGG ACG GCC TCC GAA ACC ATG GTC GAC ACG TA

98mer

Antisense primers

(9) TAC GTG TCG ACC ATG GTT TCG GAG GCC GTC CGG GGC CGG GGC TCG CGC TCC  
CTC TCC GGC TCG GAC TGC GAG GCA GCC CGC TCT CTT GCG C

94mer

(10) GCC TTG GCG GGC TCC TCT CCC TTC TGG AAC CGA GGC CCG GGC CGG GGC CTG GCT  
GCC GCT CGG CTG CCC CAA GCC TCT GCG CTG TCG ACT ACG T

94mer

notes — underlined regions indicate homology used for overlap PCR  
bold regions indicate added restriction sites (e.g. SalI)

↑ ORDER PLACED w/ BIOSOURCE INTERNATIONAL  
P.O. 941

Read & Understood by me,

str M. Mui

Invented by

Recorded by

Page No. 76

BEST AVAILABLE COPY

ESTABLISH PCR CONDITIONS FOR PCR AMPLIFYING THE  
EIA/EIB INTERGENIC REGION.

<u>Rxn</u>	<u>PRIMERS</u>	<u>TEMPLATE</u>	<u>COMMENTS</u>
1	96.74.1/2	PCITE-39	EMCV IRES 519bp
2	96.74.1/2	PCITE-39	
3	96.74.1/2	Ø	
4	96.74.3/6	CN702	Ad EIA SEQUE 288bp
5	96.74.3/6	CN702	
6	96.74.3/6	Ø	
7	96.74.4/5	CN702	Ad EIB SEQUE 396bp
8	96.74.4/5	CN702	
9	96.74.4/5	Ø	

50µl Rxn

50µM PRIMER

10mM dNTPs

1µg TEMPLATE

0.5µ DEEP  
VENT POL1µl5µl 1µl1µl0.25µlMASTERMIX

50µl BUFF

2.5µl DEEP VENT POL

10µl dNTPs (10mM)

425µl H<sub>2</sub>O

94°C X 2'  
65°C  
X { 94°C X 45"  
65°C X 30" → 55°C 1°/cycle  
72°C X 30"

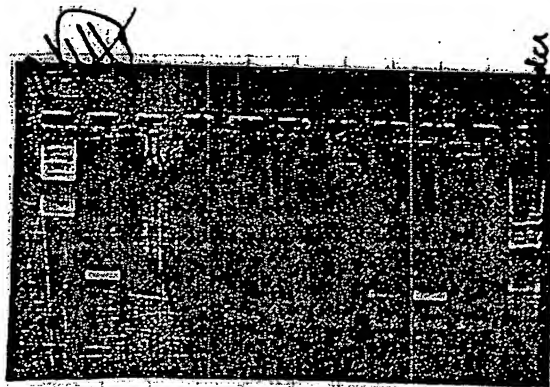
X { 94°C X 45"  
55°C X 30"  
72°C X 30"

72°C X 2'  
4°C HOLD

POSITIVE AMPLIFICATION  
PRODUCT IN LANES

1, 7, 8. AMPLIFICATION  
OF AD EIA SEQUENCE

MUST BE REPEATED. PCR MACHINE UN  
IS NOT MAKING CONTACT WITH TUBES! To Page No. 95



Issued &amp; Understood by me,

M. M.

Invented by

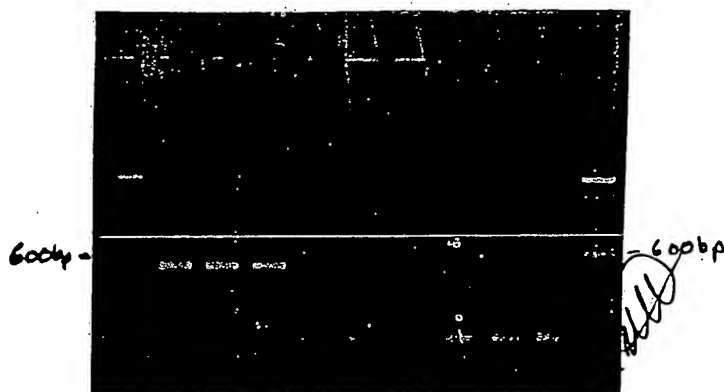
Recorded by

IRIS BEST AVAILABLE COPY  
CLONING PLATFORM  
CONSTRUCTION  
76

96

BECAUSE PCR WAS NOT OPTIMAL IN FIRST ATTEMPT  
LIKELY BECAUSE LID ON PCR MACHINE IS NOT  
MAKING GOOD CONTACT WITH TUBES, I WILL REPEAT  
RXNS FOR BETTER YIELD. SAME SETUP AS 96.76

1% AGAROSE 0.5X TAE



EXPECTED EMU IRIS PRO  
AMPLIFIED. EIA/EIB INTERG  
REGION NOT SUCCESSFULLY  
AMPLIFIED. I WILL REPEAT  
THESE REACTIONS AT 50°  
ANNEALING TEMP AND USE  
A "HOT START".

1/12/99

REPEAT PCR TO OPTIMIZE CONDITIONS - MODIFY 96.85  
BY ALTERING THE ANNEALING TEMPERATURE AND USING  
A HOT START.

RXN	PRIMERS	TEMPLATE	SIZE	MASTER 1
1	96.74.3/6	CN702	288bp	96.76 LEAVE OUT POLYMER
2	96.74.3/6	CN702	<del>296bp</del>	
3	96.74.3/6	—		
4	96.74.4/5	CN702	396 bp	
5	96.74.4/5	CN702		
6	96.74.4/5	—		

1/11/99

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OLIGOS 96.74.7 THROUGH 96.74.10 WERE RECEIVED FROM BIOSOURCE INTERNATIONAL. TO RECONSTRUCT THE MOUSE VEGF IRES DESCRIBED BY HUEZ (1998) CALLED SP163 OLIGOS 96.74.7 AND 96.74.10 WILL BE ANNEALED AND OLIGOS 96.74.8 AND 96.74.9 WILL BE ANNEALED.

~~Some~~

OLIGOS WERE REHYDRATED WITH  $H_2O$  AT  $1 \mu g/\mu l$ . SOME OF 7 AND 10 WERE MIXED AND SOME OF 8 AND 9 WERE MIXED. BOTH MIXTURES WERE DENATURED @  $100^\circ C$  FOR 10' AND ALLOWED TO COOL TO ROOM TEMPERATURE TO ACCOMPLISH ANNEALING.

11  $\mu l$  OF T4 DNA LIGASE BUFFER WERE ADDED TO EACH TUBE FOLLOWED BY 1  $\mu l$  NEB PNK (10U/ $\mu l$ ) TO PHOSPHORYLATE OLIGOS. INCUBATE @  $37^\circ C$  X 1H.

HEAT INACTIVATE PNK @  $65^\circ C$  X 20'

~~SUPPLEMENT RXN WITH~~

$\phi$ -OH/ $CHCl_3$  EXTRACT, ETOH  $\downarrow$ , RESUSPEND IN <sup>47  $\mu l$</sup>  ~~40  $\mu l$~~   $H_2O$ .  
ADD 10  $\mu l$  DNA LIGASE BUFFER AND 1  $\mu l$  T4 LIGASE.  
INCUBATE @ ~~16  $^\circ C$~~  ~~RT~~ <sup>4  $^\circ C$</sup>  ~~1H~~ <sup>O/N</sup>.

ELECTROPHORESE  
3  $\mu l$  OF UNLIGATED  
AND LIGATED  
OLIGOS TO CONFIRM  
SUCCESS OF RXNS.



SOME LIGATION  
APPEARS TO BE  
OCCURRING ILLUSTRATED  
BY THE SMEARING  
IN LANE 2. I WOULD  
HAVE EXPECTED BAND  
@  $\sim 190$ , HOWEVER.  
 $\rightarrow$  UNCATENERS CAN BE

RESOLVED BY SALT DIGEST To Page No. 89.

Issued & Understood by me.

*M. M. M.*

Invented by

Recorded by

*Andrew S. M.*

IRIS CLONING - PLATFORM  
CONSTRUCTION

96

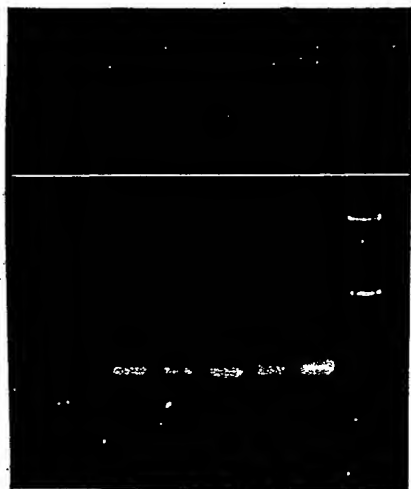
85

Rxn PROFILE

94°C X 2'  
94°C X 45"  
50°C X 30"  
72°C X 30"  
4°C HOLD

ADD POLYMERASE  
AFTER 94°C DENA  
TION.

ELECTROPHORESIS 1% AGAROSE 0.5X TAE



NO PCR AMPLIFICATION SEEN. I NEED TO  
CHECK PRIMERS TO MAKE SURE THEY  
ARE CORRECT.

-600bp

1/13/99 REPEAT PCR USING NEW TEMPLATE DNA (CN510-70.166.5)  
AND EXPAND LONG POLYMERASE

MASTERMIX #1 (DEEPVENT)

- ✓ 7µl dNTPs
- ✓ 35µl DEEPVENT BUFF.
- ✓ 298µl H<sub>2</sub>O

MASTERMIX #2 (EXPAND LONG)

- 7µl dNTPs
- 35µl EXPAND LONG BUFF.
- 298µl H<sub>2</sub>O

ADD 0.25µl PRIMERS TO EACH RXN  
ADD 0.25µl DEEPVENT OR EXPAND LONG POL  
ADD 1µl CN510 (1ng) TO EACH RXN, EXCEPT  
CONTROL

71 < 1/11/99

RXN	PRIMERS	TEMPLATE	MASTERMIX
1	96.74.3/6	CN510	#1
2	96.74.3/6	CN510	
3	96.74.3/6	Ø	
4	96.74.4/5	CN510	
5	96.74.4/5	CN510	
6	96.74.4/5	Ø	
7	96.74.3/6	CN510	
8	96.74.3/6	CN510	
9	96.74.3/6	Ø	
10	96.74.4/5	CN510	#2
11	96.74.4/5	CN510	
12	96.74.4/5	CN510 Ø	

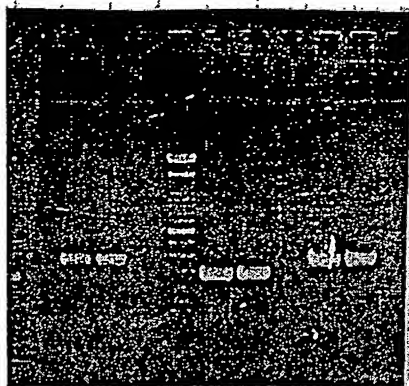
BEST AVAILABLE COPY

RXN PROFILE

94°C X 2'  
 94°C X 45"  
 55°C X 30"  
 72°C X 30"  
 4°C HOLD

"HOT START"

30X 25X



BOTH PRIMER PAIRS GIVE EXPECTED PCR AMPLIFICATION. PRODUCT (288 + 396 bp). CHANGING DNA TEMPLATE SEEMS TO HAVE MADE THE DIFFERENCE. I WILL GEL PURIFY PRODUCTS FROM DEEP VENT POLYMERASE BECAUSE OF ITS BETTER FIDELITY THAN TAQ.

be M. M.

# IRES CLONING - VEGF IRES

96

86

1/13/99

AFTER ALLOWING LIGATION TO PROCEED O/N, I  
 $\phi$ -OH/ $\text{CHCl}_3$  EXTRACTED RXN AND  $\downarrow$  DNA w/  $\text{EtOH}$ .

RESUSPEND DNA IN 75  $\mu\text{L}$   $\text{H}_2\text{O}$ . ADD 10  $\mu\text{L}$  BSI  
 ADD 10  $\mu\text{L}$  OF 10X SALT BUFFER AND 5  $\mu\text{L}$  SALT  
 (50U).

DIGEST O/N @  $37^\circ\text{C}$

ELECTROPHORESE LIGATED AND CUT SAMPLES TO  
 MONITOR PROGRESS OF RXN.



DIGESTION WITH SAL I SEEMS TO HAVE  
 RESULTED IN LOSS OF HIGHER MOLECULAR  
 WEIGHT DNA CONCATAMERS. BUT I AM  
 CONCERNED SINCE MOST OF THE PRODUCT  
 IS UNLIGATED PRODUCT. I MAY ORDER NEL  
 PNK AND/OR GEL PURIFY OLIGOS BEFORE  
 ATTEMPTING LIGATION.

2/22/99

I AM TURNING THIS PROJECT OVER TO MELISSA  
 SMITH.

*[Signature]*

*[Signature]*

from Page No. 96STRATEGY

APPRX 200 COLONIES ON EACH LIGATION  
PLATE. PICK 6 FROM EACH AND GROW  
MINIPREPS O/N IN 50  $\mu$ g/ml KANAMYCIN.

PURIFY PLASMID DNA USING RPM KIT AVAIL-  
ABLE FROM BIO L01. ELUTE DNA IN  
50  $\mu$ l OF  $H_2O$

DIAGNOSTIC DIGESTS AS FOLLOWS:

PCR BLUNT + PLATFORM 96.91

ACC/NOTI (+) 3766 bp  
416 bp  
(-) 3865 bp  
317 bp

XBAI/KPNI (+) 3417 bp  
601  
91  
73

(-) 3417 bp  
593  
93  
79

PCR BLUNT + EMCV 96.9

HINDIII (+) 3808 bp  
224 bp  
(-) 3627 bp  
405 bp

LET DIGEST PROCEED  
O/N @ 37°C

ACC/NOTI

15  $\mu$ l DNA  
2  $\mu$ l BUFFY  
2  $\mu$ l BSA  
1  $\mu$ l ENZYME TOTAL  
(5  $\mu$ l EACH)

XBAI/KPNI

15  $\mu$ l DNA  
2  $\mu$ l BUFFY  
2  $\mu$ l  $H_2O$   
1  $\mu$ l ENZYME  
(5  $\mu$ l EACH)

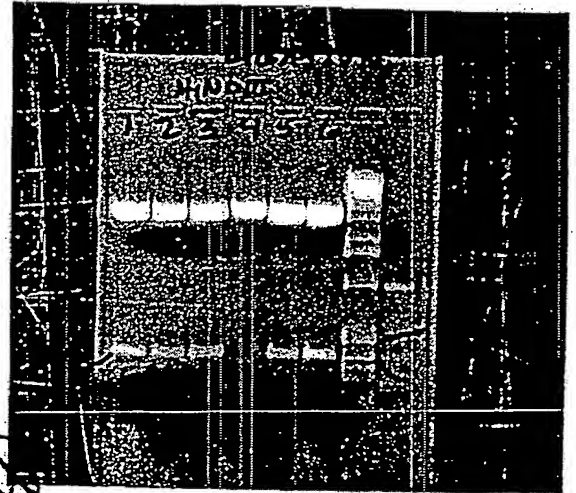
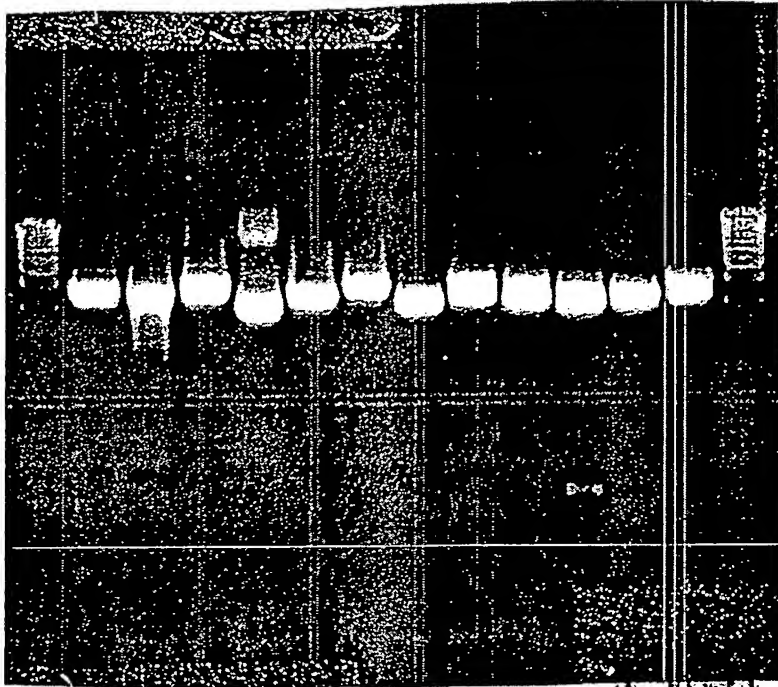
HINDIII

15  $\mu$ l DNA  
2  $\mu$ l BUFFY  
2  $\mu$ l  $H_2O$   
1  $\mu$ l ENZYME  
(5  $\mu$ l)



ELECTROPHORESIS 1% AGAROSE

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ACC/NOTI DIGEST SUGGESTS THAT CLONES 2, 3, 6 ARE RECOMBINANTS IN (-) ORIENTATION. CLONE 1 IS A RECOMBINANT IN THE (+) ORIENTATION. THE XBA/KPN1 DIGEST APPEARS TO BE A PARTIAL DIGEST. A 0.6 kb BAND IS EXPECTED, BUT THE 0.7 kb BAND IS UNEXPECTED. THE DIFFERING INTENSITIES OF THE BAND SUGGESTS THAT THIS IS A PARTIAL DIGEST ALSO. ANOTHER 0.1 kb BAND IS EXPECTED, BUT RAN OFF THE GEL. THE 0.1 kb + 0.6 kb = 0.7 kb EXTRA BAND. I WILL GROW UP MAXIS OF CLONES 1, 2 FOR ADDITIONAL CHARACTERIZATION.

CLONES 1, 2, 3, 5, 6 ARE (-) RECOMBINANTS, BUT 4 IS A (+) RECOMBINANT. I WILL GROW UP MAXI OF 1 AND 4 FOR ADDITIONAL CLONING AND CHARACTERIZATION.

done by me  
M. M. M.

Inventor by

M. M. M.

Form Page 100

INOCULATE 200ml CULTURES OF CLONES MENTIONED  
ON 96.100 WITH LEFT OVER BACTERIAL CULTURES. #  
(KANAMYCIN 50mg/ml). GROW O/N @ 37°C.

PURIFY PLASMID DNA USING QIAGEN MAXI KIT AS  
SUGGESTED BY THE MANUFACTURER. DNA W/ ISOPROPANO  
RESUSPEND IN 500µl H<sub>2</sub>O.

ELECTROPHORESE 1µl OF EACH PREP TO DETERMINE  
CONCENTRATION.



CONCENTRATION OF 96.96 1#1 = 5ng/µl  
96.96 1#2 = 200ng/µl  
96.96 2#1 = 200ng/µl  
96.96 2#4 = 200ng/µl

DIAGNOSTIC DIGESTS TO RECONFIRM STRUCTURE  
PREP DIGESTS FOR CONTINUING CLONING

KPN1/XBAI

✓ 50µl CLONES 96.96 1#1 AND #2

✓ 2µl BUFF

20 UNITS XBAI/KPN1

✓ 12µl H<sub>2</sub>O

20 µl TOTAL

SALI

✓ 50µl CLONE 96.96 1

✓ 20µl SALI BUFF

4µl SALI (80u)

20µl BSA

✓ 106µl H<sub>2</sub>O

100 µl TOTAL

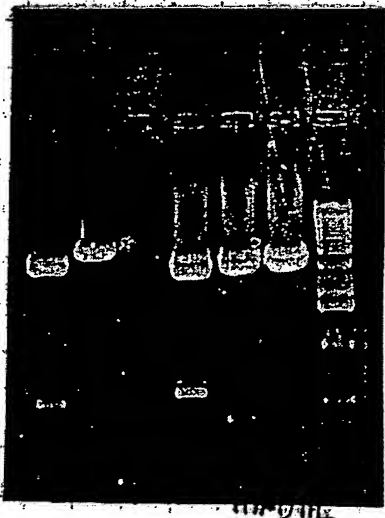
FREE STRATEGYHINDIII

✓ 5  $\mu$ l DNA CLONE 96.96 2 #1/#4  
 ✓ 2  $\mu$ l BUFF #2  
 ✓ 1  $\mu$ l HINDIII (5u)  
 ✓ 12  $\mu$ l H<sub>2</sub>O  
 20  $\mu$ l TOTAL

SALI

50  $\mu$ l DNA CLONE 96.96  
 20  $\mu$ l SALI BUF #1  
 20  $\mu$ l BSA  
 4  $\mu$ l SALI (80u)  
 106  $\mu$ l H<sub>2</sub>O  
 200  $\mu$ l TOTAL

29. ELECTROPHORESE 5  $\mu$ l OF PREP DIGESTS AND 15  $\mu$ l OF DIAGNOSTIC DIGESTS TO MONITOR RXNS. 10% AGAROSE.



DIGESTS YIELD EXPECTED RESULTS AS INDICATED ON PHOTOGRAPH. SALI DIGESTS OF VECTOR (96.96 1 #2) AND INSERT (96.96 2 #1) WERE SUCCESSFUL. NEXT STEP IS TO CIP VECTOR AND THEN GEL PURIF. BOTH VECTOR + INSERT PRIOR TO LIGATION.

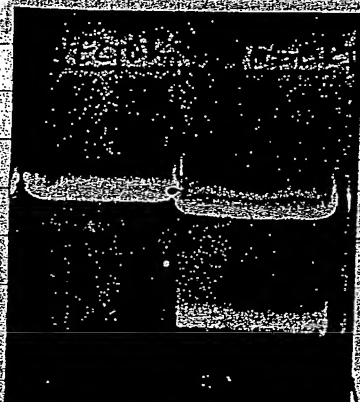
96.96.1 #2 = CP 624  
 96.96.2 #1 = CP 625

O-H/ CHCl<sub>3</sub> EXTRACT VECTOR, PRECIPITATE w/ 3 VOLUMES ETH. RESUSPEND PELLET IN 90  $\mu$ l H<sub>2</sub>O. ADD 10  $\mu$ l OF NEB BUFFER 3. ADD 2  $\mu$ l CIP. INCUBATE RXN FOR 1h @ 37°C. STORE @ -20°C UNTIL PURIFICATION.

with M. M.

From Page No. 102

GEL PURIFY INSERT AND VECTOR - 1% AGAR



EXTRACT BANDS, PURIFY DNA  
USING QIAGEN QIAEXII KIT  
ELUTE w/ 10ul H<sub>2</sub>O.

ELECTROPHORESE 1ul 0.8%  
AGAROSE TO DETERMINE YIELD



[VECTOR] = 20 ng/μl  
[INSERT] = 5 ng/μl

### LIGATION SETUP

A  
✓ 2ul VECTOR  
✓ 2ul INSERT  
1ul LIGASE  
1ul BUFF  
1ul H<sub>2</sub>O

B  
✓ 2ul VECTOR  
1ul LIGASE  
1ul BUFF  
6ul H<sub>2</sub>O

C  
✓ 2ul INSERT  
1ul LIGASE  
1ul BUFF  
6ul H<sub>2</sub>O

16 °C O/N

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DILUTE LIGATIONS 1:10 IN  $H_2O$ . TRANSFORM DH10 IS WITH 1  $\mu$ l BY ELECTROPORATION. ALLOW TO RECOVER FOR 45' @ 37°C. PLATE ON KAN PLATES INCUBATE @ 37°C O/N.

1  $\mu$ l COLONIES ON PLATE A. AND B. DIFFICULT TO DETERMINE IF THERE IS ANY DIFFERENTIAL NO COLONIES ON PLATE C. PICK 12 COLONIES FROM PLATE A. GROW O/N IN KAN/LB.

PURIFY PLASMID DNA FROM MINICULTURES USING BIO 101 RPM KIT (LOT # 2070-600-536-3). ELUTE DNA w/ 50  $\mu$ l  $H_2O$ .

DIAGNOSTIC DIGESTS w/ HINDIII TO DETERMINE RECOMBINANTS.

#### EXPECTED DIGESTS:

PLATFORM + IRES (+) ORIENTATION	3.9 kb
	0.8 kb
PLATFORM + IRES (-)	4.1 kb
	0.6 kb

\*N  
ETUP → 15  $\mu$ l DNA  
2  $\mu$ l NEB 2  
3 UNITS HINDIII  
3  $\mu$ l  $H_2O$

DIGEST FOR 5 hrs @ 37°C

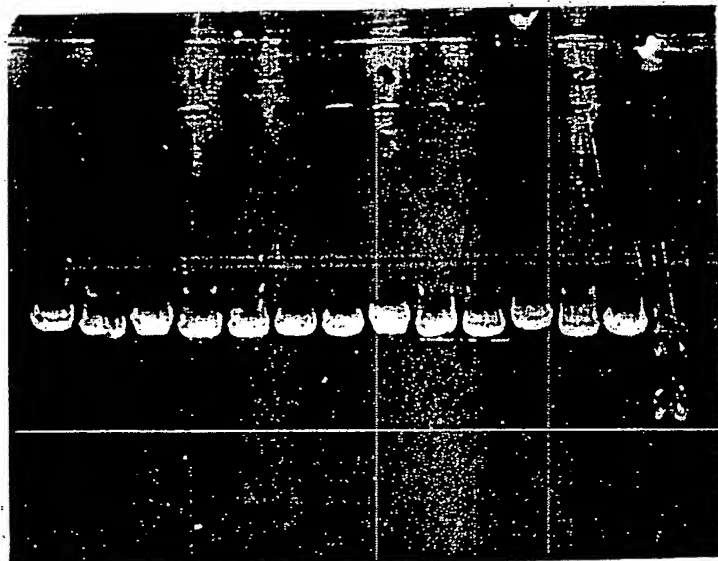


# PLATFORM-IRE CLONING

104

104

ELECTROPHORESE SAMPLES - 0.8% AGAROSE GEL 70V  
0.5X TAE

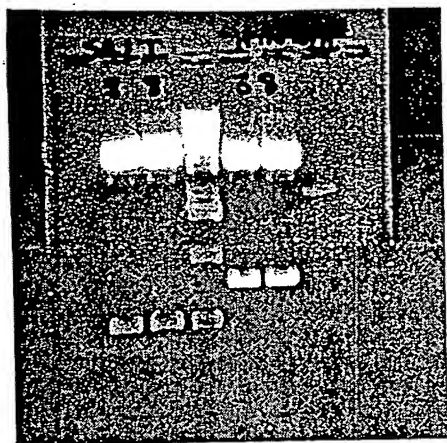


CLONES 3 AND 8 ARE  
RECOMBINANTS. HOWEVER  
SINCE RESOLUTION IS  
POOR, I CANNOT DETER-  
MINE ORIENTATION.  
I WILL REPEAT THIS  
DIGEST TO CONFIRM  
CLONES.

VECTOR CONTROL  
YIELDED LINEARIZED  
PRODUCT 2.4kb AS  
EXPECTED.

REDIGEST CLONES 3  
8 WITH HINDIII (LIKE  
AND SALI (96.101) 5h  
@ 3:

ELECTROPHORESE - 1%  
AGAROSE, 0.5X TAE



SALI DIGEST, 3 + 8 ARE AS  
EXPECTED (INSERT @ 0.5kb). HINDI  
DIGEST IS CONSISTENT W/ (+)  
ORIENTATION RECOMBINANT. I WILL  
STREAK 3 + 8 ON PLATE  
AND GROW MAXI PREPS PRIOR  
TO SEQUENCING.

96.103.3 = CP626

A. A. C. A. K. H.

IRES CLONING → CN306

96

105

2 200ml CULTURES INOCULATED (50µg/ml KAN).  
GROW O/N @ 37°C

PURIFY 96.103.3 AND 96.103.8 DNA USING  
QIAGEN MAXIPREP KIT. ELUTE DNA IN 500µl H<sub>2</sub>O.  
STORE @ -20°C UNTIL NEEDED.

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BEGIN CLONING OF PLATFORM PLASMID  
CONTAINING THE EMCV IRES INTO CN306 BACKBONE

DIGEST 96.103.3 AND CN306 (96.98) WITH KPN I  
AND XBA I.

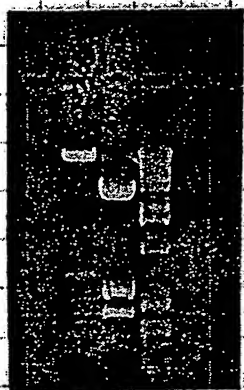
96.103.3 (6PG26)  
✓ 50 µl DNA (96.107) 10 µg  
✓ 20 µl BUFF #2  
4 µl XBA (80 U)  
✓ 4 µl KPN I (80 U)  
✓ 122 µl H<sub>2</sub>O

CN306  
✓ 50 µl DNA (96.98) -1  
✓ 20 µl BUFF #2  
4 µl XBA (80 U)  
✓ 4 µl KPN I (80 U)  
✓ 122 µl H<sub>2</sub>O

DIGEST 4h @ 37°C

Andrew G. Hill

ELECTROPHORESE 10  $\mu$ l OF EACH RXN TO MONITOR PROGRESS OF RXN.



CN306 DIGEST IS COMPLETE.  
HOWEVER, 96.103.3 DIGEST PRODUCED UNEXPECTED RESULTS. AFTER INSPECTION OF ORIGINAL CONSTRUCT, I REALIZED THAT 96.103.3 HAS A KPN1 SITE IN THE DREZ THAT I HAD NOT ACCOUNTED FOR. EXPECTED BANDS ARE, THEREFORE,

	3.4 kb
	0.6
	0.47
	0.09
	0.07

RESULTS ARE CONSISTENT.

TO GET AROUND THIS PROBLEM, I WILL DO A PARTIAL KPN1 DIGEST AND GEL PURIFY 1kb BAND.

DIGEST 96.103.3 WITH XBA1 COMPLETELY.  
FOLLOW UP WITH PARTIAL KPN1 DIGEST TOMORROW.

130  $\mu$ l DNA (26  $\mu$ g)  
20  $\mu$ l BSA  
20  $\mu$ l BUFF 2  
6  $\mu$ l XBA1 (120 UNITS)  
24  $\mu$ l H<sub>2</sub>O

DIGEST O/N @ 37°C



108

DIVIDE XBAI DIGEST INTO 3, 65  $\mu$ l/TUB  
DIGEST EACH TUBE FOR 1h @ 37°C AS  
BELOW:

#1  
20 UNITS KPN1

#2  
10 UNITS

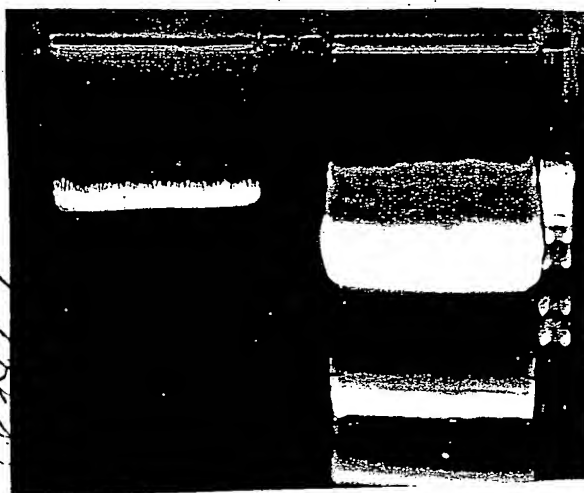
#3  
5 UNITS

AFTER RXN INCUBATION, PLACE TUBES ON ICE  
ELECTROPHORESE 5  $\mu$ l TO MONITOR RXN  
PROGRESS.



*am*

PARTIAL DIGEST WAS SUCCESSFUL.  
~1kb BAND IS PRESENT. NEXT STEP  
IS TO PURIFY BAND BY ELECTROPHORESIS



PURIFY DI  
BY QIAEX  
KIT-ELUT  
W/ 10  $\mu$ l

1kb



*am*

DETERMINE [DNA]  
PRIOR TO LIGATION

$$[96.103.3] = 20 \text{ ng/l}$$

$$[\text{VECTOR}] = 10 \text{ ng/l}$$

11

Andrew S

10.11.11

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LIGATION

<u>A</u>	<u>B</u>	<u>C</u>
✓ 5µl VECTOR (50ng/µl)	✓ 1µl FRES INSERT	✓ 5µl VECTOR
✓ 1µl FRES INSERT	✓ 1µl BUFF	✓ 1µl BUFF
✓ 1µl BUFF	✓ 1µl LIGASE	✓ 1µl LIGASE
✓ 1µl LIGASE	✓ 7µl H <sub>2</sub> O	✓ 3µl H <sub>2</sub> O
✓ 2µl H <sub>2</sub> O		

INCUBATE RT X 2hr

TRANSFORM LIGATIONS (DILUTED 1:10) DH10B ELECTRO  
COMPETENT CELLS. PLATE ON LB/AMP PLATES.  
INCUBATE O/N.

TMC COLONIES ON PLATES A AND C. PICK  
10 FROM PLATE A FOR MINIPREPES. GROW O/N  
@ 37°C.

PURIFY PLASMID DNA USING RPM BIO101 KIT.  
ELUTE PLASMID DNA IN 50µl OF H<sub>2</sub>O.  
CHARACTERIZE RECOMBINANTS WITH KPN1 AND  
HINDIII DIGESTS.

*(Signature)*

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## DIGEST SET UP: SCREEN FOR RECOMBINANTS.

KPN I

15  $\mu$ l DNA  
 2  $\mu$ l BSA  
 2  $\mu$ l BUFF 2  
 1  $\mu$ l enzyme (34)

HIND III

15  $\mu$ l DNA  
 2  $\mu$ l GUF 2  
 1  $\mu$ l enzyme (34)  
 2  $\mu$ l H<sub>2</sub>O

DIGEST 4 hrs @ 37°C

ELECTROPHORESIS @ 90V, 0.8% AGAROSE 1hr

KPN

HIND III

2.0  
1.6

## EXPECTED BANDS:

HIND III - 8.3 kb  
 1.4 kb

KPN I - 9.3 kb  
 0.5 kb

CM306 VECTOR SHOULD BE LINEARIZED IN EACH CASE

KPN I DIGEST DOES NOT APPEAR COMPLETE. HOWEVER, HIND III DIGEST INDICATES THAT CLONES 96.115 1, 2, 3, 4, 5, 6, MAY BE RECOMBINANTS. ~~2~~ BAND @ 1.5 kb IS INDICATIVE. CLONES 1 AND WILL BE MAXIPREPED, AND THEIR STRUCTURE WILL BE CONFIRMED WITH ADDITIONAL DIGESTS BEFORE CONTINUING.

NOTE: THIS CLONING IS BEING TURNED OVER TO MELISSA SCOTT

TITLE EMCV DRES CLONING

Proj. No. \_\_\_\_\_

Book No. 96From Page No. 116

INOCULATE 250 ml LB/AMP WITH BACTERIA FROM 96.11  
AND 96.115-2 GROW O/N @ 37°C SHAKING

PURIFY PLASMID DNA USING QIAGEN MAXIPREP KIT  
AS SUGGESTED BY MANUFACTURER. ELUTE RESUSPEND  
DNA IN 500 µl H<sub>2</sub>O

CLONING TURNED OVER TO MELISSA SCOTT.

*[Signature]*

Witnessed &amp; Understood by me,

Invented by

Page No. X

From Page No. \_

GOAL → to regulate the expression of both the E1A gene and the E gene by using a single tissue specific promoter. To do so an internal ribosome entry site (IRES) must be cloned b/t the 2 g  
↳ an internal entry point for initiation of translation by euk. ribosomes

## 2 Potential IRES:

- ① EMCV: encephelomyocarditis virus. Well characterized. Commercially available from Novagen. (450bp) efficient translation. bicistronic message, commonly used in gene therapy.
- ② VEG-F: from mouse, vascular endothelial growth factor. Active in hypoxic environments, 1000nt in length

## Advantages of including IRESes in ARCA system

- regulate expression of two Ad genes from a single tissue specific
- regulate expression of one Ad gene and one cytotoxic gene (CD, P450, IL4) from a single TRE
- prevent homologous recombination
- preferential expression of IRES controlled gene in hypoxic cells

Want to design a platform plasmid for constructing Ad mutant that contain IRESes.

Picking up project in the beginning → where Andy left off.

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Witnessed &amp; Understood by me,

Heather Connors

Invented by

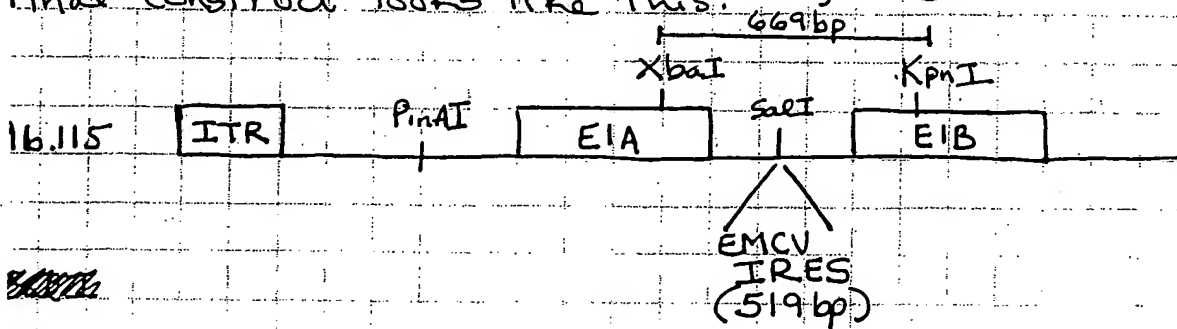
11/1/11

Page No. \_

From Page No. 114

Andy created the following deletion in CN306 (derived from pXC.1)  
 ↳ lacks the E1a promoter area

Deleted the E1B promoter and E1a poly a signal, but leaves the E1a + E1b coding regions intact. Final Construct looks like this:



used overlapping PCR to delete E1B promoter poly a signal at the same time introduced a SalI site for insertion of IRESes

Andy → the EMCV IRES was amplified from pCITE3 (Novagen) w/ SalI ends that were used for ligation into the platform plasmid.

The IRES insert will be 6 bases upstream of the E1B translation initiation codon.

The PCR product was then cloned back into CN306 ↑ XbaI / KpnI

The EMCV IRES contains a pyrimidine rich tract of bases near its 3' end that are imp. for its activity. This pyrimidine tract will be 27 bases from the E1b initiation codon.

This is where Andy is at. He would like me to confirm the clone which is the platform plasmid + EMCV IRES. This clone is called 96.115 for now.

Once clone is confirmed want to add 2 different promoters:

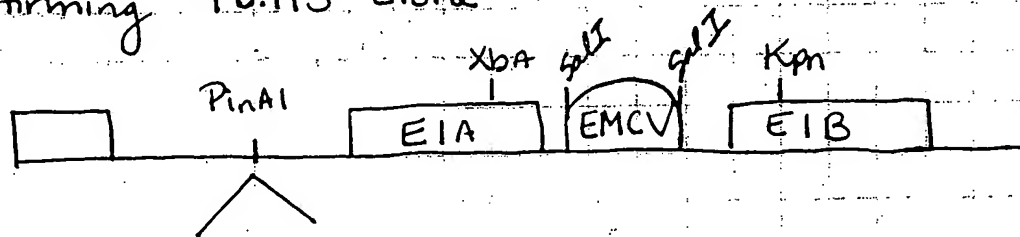
- ① PB: probasin promoter, prostate. Can obtain from CP251 w/ PnAI.
- ② CMV: cytomegalovirus promoter, expressed 100 fold more. Need to amplify w/ PnAI sites 5' + 3'

CPV787 has prostate specific promoter. CMV active in all cells. Very Active (100 fold more)



Page No. 115

Confirming 96.115 clone



- ① PB promoter
- ② CMV promoter

CN251 TPinAI fragment  
design primers for PCR TPinAI 5' and 3'

1. Digest: Andy has 2 potential clones that need to be confirmed

2. Sets of Digests:

**Xba/KpnI** buffer 2 + BSA

CP306: 8600 bp, 700 bp

96.115 #1 + #2: 8656 bp, 641, 467

**KpnI** buffer 1 + BSA

CP306

96.115 #1 + #2

**Cla/AvrII** buffer 4 + BSA

CP306

9.4 Kb, linear no AvrII

96.115 #1 + #2

**PinAI** Gibco buffer 4. Doing this digest now in case clones are correct then can proceed right away w/ ligating PB promoter into plasmid construct.

CP251 DNA from DC 1.15 µg/µl (used 2 µl for digestion)

To Page No. 11

Witnessed &amp; Understood by me,

Heather Connors

Invented by

Recorded by

Heather Connors

TITLE \_\_\_\_\_

Proj. No. \_\_\_\_\_

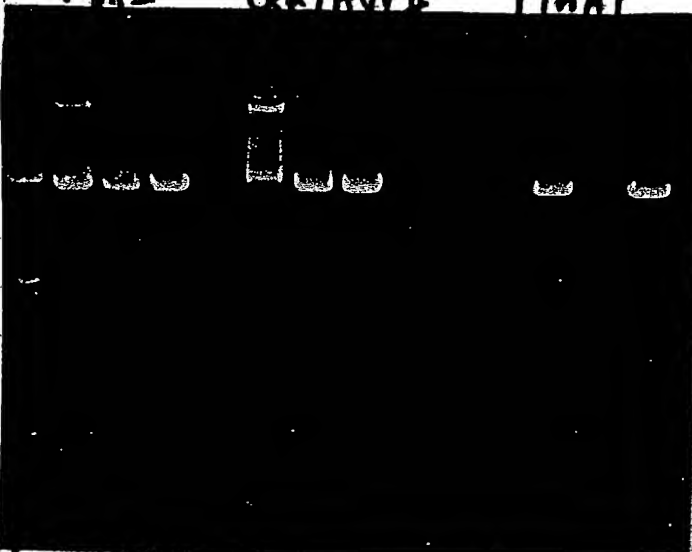
Book No. 99From Page No. 116

Xba/Kpn

KpnI

Cla/AvrII

PnaI

Results

Xba/Kpn: <sup>CP</sup>306 dropped the correct size fragment → 700bp  
 9b.115#2 is correct. Think #1 is correct too - just difficult to see bands in the ~~gel~~ dye bands.

Cla/AvrII: ClaI is not cutting due to dam methylation!!  
 Overlooked this. ∴ CP306 is uncut. #1 + #2 are methylated by AvrII

PnaI: #1 + #2 look OK. CP251 is not 1.15 µg/µl!

Next: believe #2 looks correct. WANT to be sure.  
 Use more DNA + rpt digests: Digest all 3 constr.

① Xba/KpnI buffer 2 + BSA: 10 µl DNA

② Hind III 10 µl DNA  
 Looking for 1.4 and 8.3 Kb band.

CP251: inoculated o/n culture from glycerol stock LB+



Form Page No. 11

CP 306

96.115 #1

96.115 #2

Xba/Kpn I

buffer 2

BSA

H3

buffer

2

DNA I10 $\mu$ l (~2 $\mu$ g)  
10 $\mu$ l (~2 $\mu$ g)

a) 37°C 9AM -

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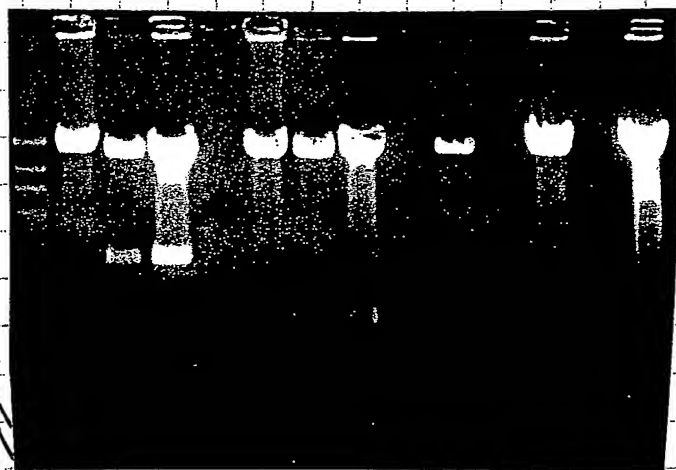
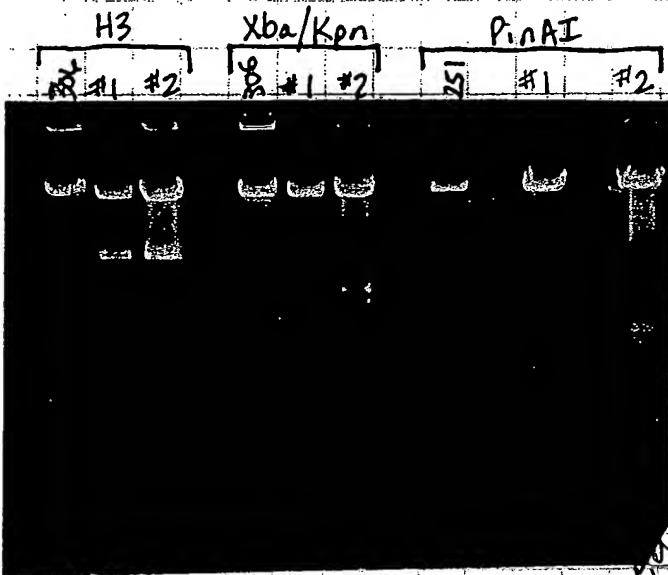
Also repeated PinAI digest.

2 mini-preps of CP251. Will ~~add~~ 1 tube to company DNA stock. (-20)

Digested the following samples w/ PinAI:

CP251 25 $\mu$ l DNA (~2.5 $\mu$ g)  
3 $\mu$ l Gibco buffer 4  
2 $\mu$ l PinAI (10 units)  
30 $\mu$ l96.115 #1+2 20 $\mu$ l (~4.0 $\mu$ g)  
5 $\mu$ l H<sub>2</sub>O  
3 $\mu$ l Gibco buffer 4  
2 $\mu$ l PinAI  
30 $\mu$ l

b) 37°C 9:30 -



Results: MARKER looks terrible. Try using Andy's 1Kb ladder.  
 HARD to tell size of H3 Fragments. Need to rpt digest! (aga)  
Xba/Kpn → fragments so faint but both look OK.  
PinAI: Andy thinks maybe there is not enough DNA for 251  
 so 500 bp fragment isn't showing up.

To Page No. 11

Witnessed &amp; Understood by me,

Date

Invented by

Recorded by

Heather Connors

Melissa J. [Signature]

From Page No. 118

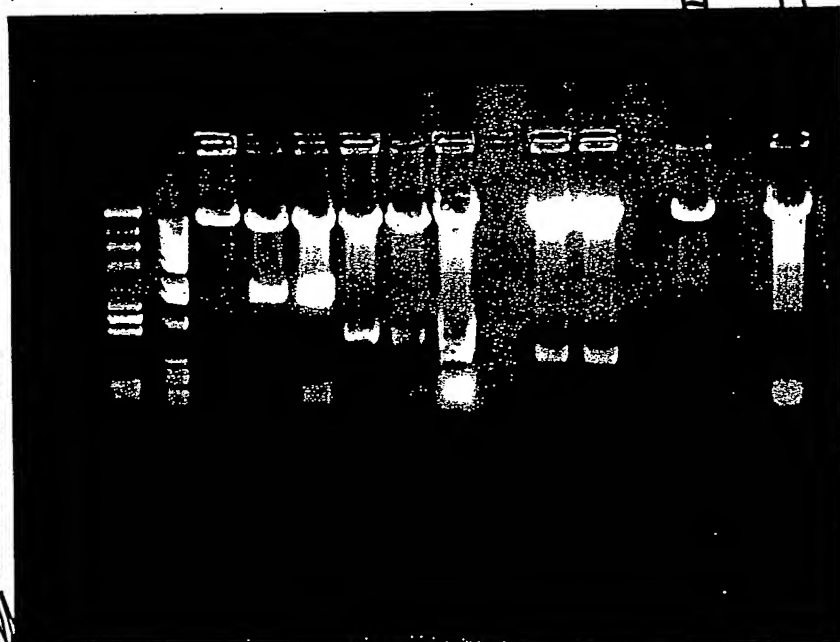
Next: Do maxi-prep for 251 FINAL Volume 400 $\mu$ l

Then set up the following o/n digestions: (maybe digestion will be more com)

 $\uparrow$  H3 buffer 2: 10 $\mu$ l DNA CP306, 96.115 #1 + #2 $\uparrow$  Xba/Kpn buf 2 + BSA: 10 $\mu$ l DNA CP306, 96.115 #1 + #2

PinAI: 96.115 #1 + #2: 25 $\mu$ l DNA  
 3 $\mu$ l buffer:  
 2 $\mu$ l enzyme  
 30 $\mu$ l

CP251: 100 $\mu$ l DNA (~20 $\mu$ g)  
 12 $\mu$ l buffer  
 8 $\mu$ l PinAI (40 Units)  
 120 $\mu$ l



@ Purified: CP251 500bp fr  
 96.115  $\uparrow$  PinAI #1 9Kb  
 #2 9Kb

CIP Treated Both #1 and #2  
 (used same protocol as on p. 35)

17 $\mu$ l DNA (gel purified)  
 2 $\mu$ l buffer  
 1 $\mu$ l CIP (Boehringer)  
 20 $\mu$ l

@ 37°C 1h

Added 0.2 $\mu$ l of .5M EDTA

@ 75°C 10 min

Phenol / CHCl<sub>3</sub>

EtoH ppt (15 $\mu$ l)

Resuspend in 15 $\mu$ l H<sub>2</sub>O

CP627: 96.115 #2

★ See page 135

Witnessed & Understood by me:

Heath: (for now)

Invented by

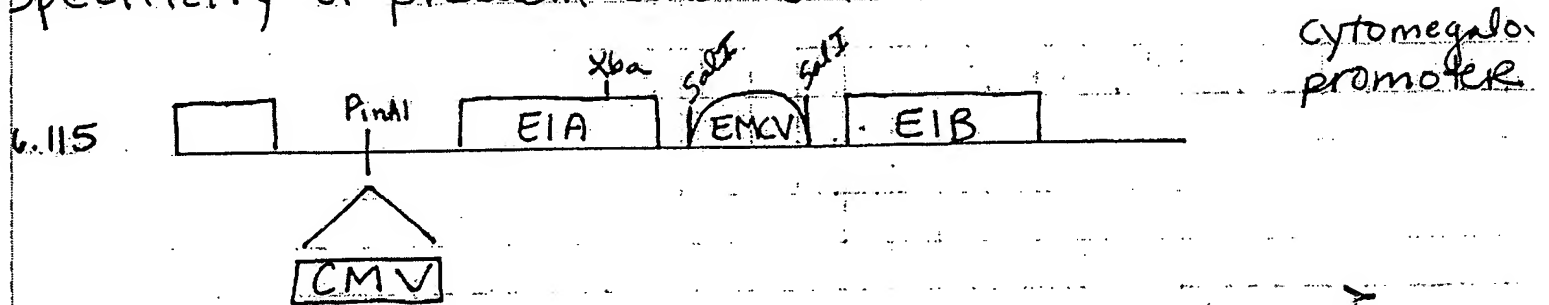
Records: [Signature]

To Page 1

Page No.     

Goal → also want to add CMV promoter to platform construct.  
 PCR promoter + clone into PinAI site of 96.115

CMV promoter will act as a control → the probasin promoter is specific to the prostate. CMV is active in many different types of cells. Can use these 2 for comparison → look at specificity of probasin construct.



## Primer Design

2/23/99

99.120.1 FW Primer PinAI (Anneals to 27-41 in pCMVB, Clontech)  $T_m = 40C$

5'-ACG TAC ACC GGT CGT TAC ATA ACT TAC -3'

99.120.2 RV Primer PinAI (Anneals to 535-548 in pCMVB, Clontech)  $T_m = 42C$

5'-CTA GCA ACC GGT CGG TTC ACT AAA CG-3'

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Witnessed &amp; Understood by me,

Heather Connors

Invented by

Recorded by

 To Page No. 1

Project No. \_\_\_\_\_  
Book No. 99  
TITLE Ligation of PB into Platform Construct

From Page No. 119

Ligation Platform Construct + Probasin promoter  
4  $\mu$ l vector (CIP treated 96.115)  
6  $\mu$ l fragment (CP251 + PinAT)  
12  $\mu$ l buffer  
8  $\mu$ l ligase  
2.0  $\mu$ l  
@ 16°C o/n

Transform JM109 180  $\mu$ l + 12  $\mu$ l ligation rxn LB+Amp

Colonies w/ both constructs  
Stored plates at 4°C  $\rightarrow$  pick colonies 2/28/99

Picked 16 colonies (96.115 #2 + PB promoter)  
4ml LB+Amp @ 37°C o/n

Mini-Preps

R. Digest NruI / BclI Expected fragment size: 6334,  
~~6334~~  $\rightarrow$  unique in PB promoter

10  $\mu$ l DNA  
2  $\mu$ l buffer 7 Gibco  
1  $\mu$ l NruI

6  $\mu$ l H<sub>2</sub>O  
19  $\mu$ l @ 37°C 1hr  
heat inactivate @ 65°C 20min  
add 1  $\mu$ l BclI @ 50°C 1hr



Run gel longer  $\rightarrow$  see page 129

Page No. 132

10

Transformation very efficient - maybe self-ligation.  
Check 9 transformants for exact clone.

Inoculated 4ml LB + Amp @ 37°C O/N

- ① #1-9 CMV + CP306  
② #10-18 PB + CP306

Mini-preps

R. Digest

↑ PstAI buffer 4 Gibco

20 µl DNA

2.5 µl Gibco 4

2.5 µl PstAI (12.5 U)

25.0 µl

@ 37°C

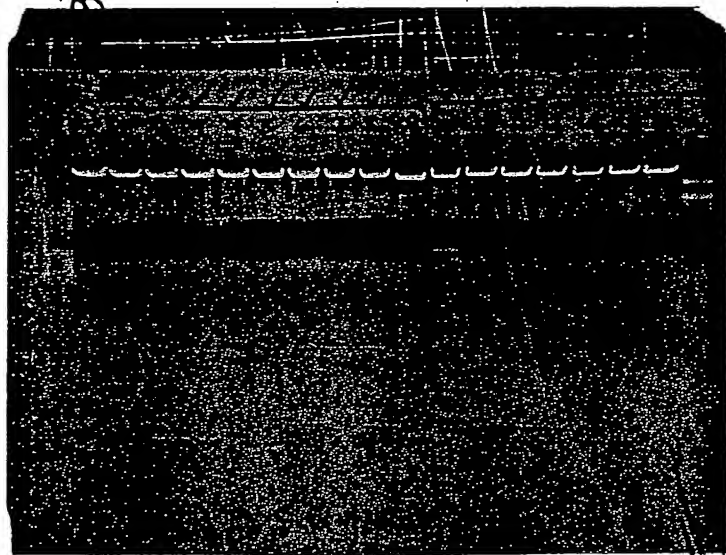
10:30 - 1:30 pm

#18 did not fit on gel!

Results:

CMV 8/9 correct  
PB 6/8 correct

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Page No. \_\_\_\_\_

Witnessed & Understood by me,

Invented by

Recorded by

Carly Corrao

*[Signature]*

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firm Orientation of the following clones:

~~CP1506~~ + CMV # 1, 4, 5, 7, 17

~~CP1506~~ + PB # 2, 3, 5, 6, 7, 8, 13, 14, 16

same digests as before for screening: (p130)

CMV Constructs: Sst/H3 buffer 2

PB Constructs: ~~MS~~ H3 buffer 2 + ~~MS~~

@ 37<sup>10AM</sup> - 4pm

15  $\mu$ l DNA  
1  $\mu$ l enzyme  
1  $\mu$ l enzyme  
2  $\mu$ l buffer  
20  $\mu$ l

1)  $\uparrow$  Sst/H3 buffer 2

ROL 96.115

5480

2003

1030

841

410

Negative Orientation

4290

2003

1871

1718

410

Positive

4780

2003

1228

1030

841

410

BEST AVAILABLE COPY

$\uparrow$  H3 buffer 2

Negative Orientation

7603

1440

1219

Positive Orientation

7155

1677

1440

CONTROL

8362

1440

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Read & Understood by me,

*after Conours*

Invented by

Recorded

*[Signature]*

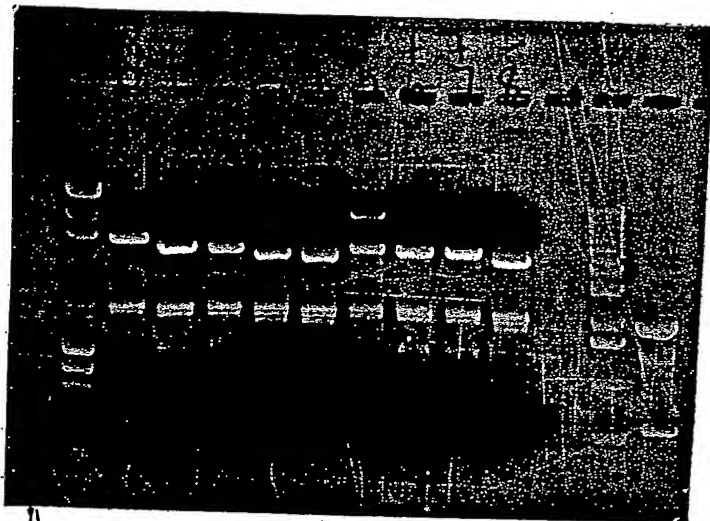
TITLE

CP 632 , CP 633

Project No.

Book No.

From Page No.



Results: 5/8 CORRECT orientation

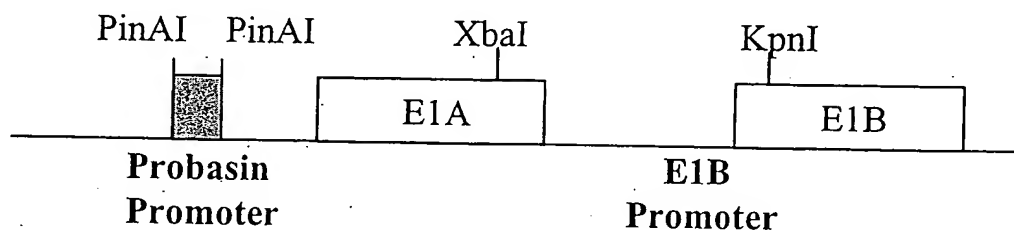
#1, 2, 5, 4, 7

CP633

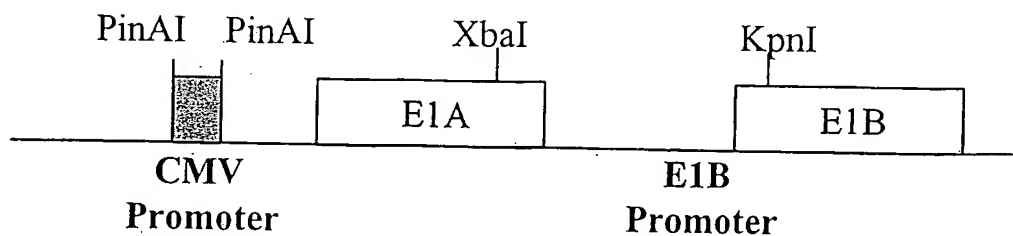
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Control Constructs

**CP632:** CP306 + Probasin promoter obtained from CP251 digested with PinAI. Control Protein- **E1B** promoter and E1A poly A signal present, no IRES.

#10  
99.137Glycer  
Stocks  
Prepared  
3/16

**CP633:** CP306 + CMV promoter amplified from pCMVbeta (Clontech) with PinAI 5' and 3' ends for ligation. Control Protein- **E1B** promoter and E1A poly A signal present, no IRES.

#1  
99.139

Witnessed &amp; Understood by me.

Date

Invented by

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